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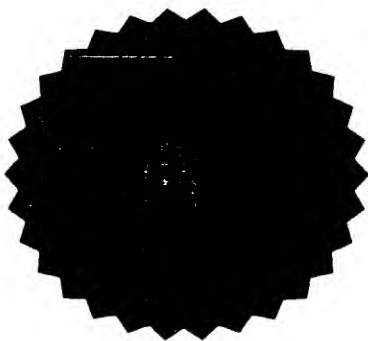
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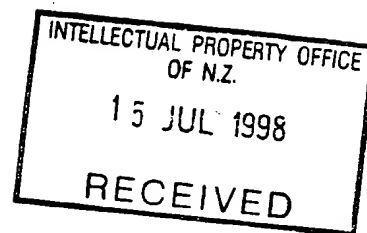
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**NEW ZEALAND
PATENTS ACT, 1953**

PROVISIONAL SPECIFICATION

**IMPROVEMENTS IN OR RELATING TO CHIMERIC POLYPEPTIDES OF
BIOTIN BINDING PROTEINS**

WE, JOHN CRISTELLER, a New Zealand citizen of 492 College Street, Palmerston North, New Zealand; PAUL SUTHERLAND, a New Zealand citizen of 22 Royal Terrace, Sandringham, Auckland, New Zealand; COLLEEN MURRAY, a New Zealand citizen of 6 Williams Terrace, Palmerston North, New Zealand; NGAIRE MARKWICK, a New Zealand citizen of 21 Lingham Crescent, Torbay, Auckland, New Zealand; and MARGARET PHUNG, a New Zealand citizen of 29 Juliana Place, Palmerston North, New Zealand, do hereby declare this invention to be described in the following statement:

IMPROVEMENTS IN OR RELATING TO CHIMERIC POLYPEPTIDES OF BIOTIN BINDING PROTEINS

FIELD OF THE INVENTION

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This invention relates to chimeric polypeptides comprising vacuole targeting sequence and biotin binding sequences. The polypeptides are useful in methods for conferring pest resistance on plants and in the production of compositions useful as pesticides. The methods and compositions form further aspects of the invention.

10

BACKGROUND OF THE INVENTION

15 Pests such as insects, nematodes and mites are a significant economic cost to plant-based industries. Losses arise through production lost to pest consumption, spoilage and introduction of disease carried by pests.

Traditionally, control of pests has been pursued through the application of pesticide chemicals. Continued use of chemicals is subject to a number of disadvantages. Pests can develop tolerance to chemicals over time producing pesticide resistant population.
20 Chemical residues may also pose environmental hazards as well as health concerns.

Biological control presents an alternative means of pest control which is potentially more effective and specific than current methods, as well as reducing dependence on chemical pesticides. The need for biological controls has led to the use of recombinant DNA techniques to insert genes which express pesticidal toxins into plant cells.
25

This technology in turn may also give rise to resistant pest populations. There is therefore an ongoing need to find proteins with pesticidal properties, particularly those that are encoded by single genes. These genes can be used to transform plants to produce pest resistant cultivars.
30

Genes studied to date include a range of *cry* genes from the bacterium *Bacillus thuringiensis* (Bt) encoding β -endotoxins and various higher plant genes encoding antimetabolites such as protease and α -amylase inhibitors and lectins (Boulter, 1993).
35 Many transgenic cultivars with improved insect resistance are now being commercialised for example, transgenic cotton, corn, and potatoes (James and Krattinger, 1996). More recently, the use of avidin and streptavidin as larvicides against insect pests has been

explored (WO 94/00992 and Morgan et al.; 1993). Generation of resistant plants has been sought by inserting into the cells of a plant a gene whose expression causes production of one or more of those glycoproteins in larvicidal amounts. To date, limited success has been achieved in producing insect resistant plants using this technology.

The commercial production of avidin from reproductive tissue of plants using such constructs has also been contemplated (WO 97/17455). The production methods are subject to a number of drawbacks. Male fertility in plants can be lost and expression in vegetative tissue may be low. This may be due in part to expression being outside the cell.

It is an object of the present invention to provide chimeric polypeptides which go some way to overcoming the above drawbacks or at least to provide the public with a useful choice.

SUMMARY OF THE INVENTION

Accordingly, in one aspect, the present invention may be broadly said to consist in a chimeric polypeptide that comprises (a) a vacuole targeting sequence encoding a polypeptide; and (b) a sequence encoding a biotin-binding protein linked in operable combination to said targeting polypeptide.

Preferably, the vacuole targeting polypeptide is a signal sequence polypeptide selected from proteinase inhibitor signal sequence (PPI-I or PPI-II) polypeptide which have the amino acid sequences set out in Figure 8b and Figure 9b respectively, or variants thereof having substantially equivalent signaling activity thereto. Numerous other signal sequences which carry out this function are described in the literature and might also be used.

Preferably, the biotin-binding protein encoded is avidin or streptavidin or a functionally equivalent variant thereof.

Conveniently, the chimeric polypeptides of the invention are obtained by expression of a DNA sequence encoding the chimeric polypeptide in a host cell or organism.

In a further aspect, the present invention provides an isolated nucleic acid molecule encoding a chimeric polypeptide of the invention.

This nucleic acid molecule can be an RNA or cDNA molecule but is preferably a DNA molecule.

Also provided by the present invention are recombinant expression vectors which contain a DNA molecule of the invention, and hosts transformed with the vector of the invention capable of expressing a polypeptide of the invention.

In a still further aspect, the invention provides a method of producing a polypeptide of the invention comprising the steps of:

10

(a) culturing a host cell which has been transformed or transfected with a vector defined above to express the encoded polypeptide of the invention; and optionally

(b) recovering the expressed polypeptide.

15

An additional aspect of the present invention provides a ligand that binds to a polypeptide of the invention. Most usually, the ligand is an antibody or antibody binding fragment.

In a further aspect, the present invention provides a method for producing a pest resistant plant, comprising transforming the plant genome to include at least one DNA molecule of the invention.

The present invention further provides a transgenic plant that contains a DNA molecule of the invention.

25

In a still further aspect, the present invention provides a method for killing pests comprising administering to said pest an amount of a chimeric polypeptide of the invention effective to kill said pest.

30 Usually, the pests are insect larvae.

In yet a further aspect, the present invention provides a composition comprising a chimeric polypeptide of the invention and a carrier, diluent, excipient or adjuvant.

35 The composition is preferably a pesticidal composition.

In a still further aspect, the present invention provides a method for producing avidin

streptavidin, the method comprising extracting avidin or streptavidin from a pl containing a DNA molecule of the invention coding for avidin or streptavidin.

While the invention is broadly as defined above, it will be appreciated by those pers
5 skilled in the art that it is not limited thereto and that it also includes embodiments
which the following description gives examples.

Figure 1 shows the nucleic acid sequence of Potato Proteinase Inhibitor I (PPI-I/pUC1
The signal sequence is in bold type and the start and stop codons are in italic. T
10 mutagenic primer is denoted by underlined in lower case with the Bgl II site created
mutagenesis in bold italic. The upstream and downstream primers used were the Forw
and Reverse M13(lacZ) Primers [Perkin Elmer].

Figure 2 shows Avidin cDNA (pGEMav). The signal sequence represented in bold ty
15 start and stop codons are in italic, primers are underlined lower case with the BamH I s
created by mutagenesis in italic. The downstream primer used was the Reve
M13(lacZ) Primer [Perkin Elmer].

Figure 3 shows streptavidin cDNA (Streptavidin/pUC19). Start and stop codons are
20 bold type. EcoR I and Xba I sites are in italic.

Figure 4 shows potato proteinase inhibitor II (PPI-II/pUC19). The signal sequence
represented in bold type and start and stop codons are in bold italic. Underlined ty
denotes the intron within the signal sequence. The asterisk denotes the result of PCR en
25 during isolation of the PPI-II sequence.

Figure 5 shows components of the ligation reaction to produce recombinant pAR
containing the PPI-I signal sequence/Avidin cDNA gene fusion. A) PPI-I leader fragme
resulting from a Sal I/Bgl II digest of the mutated PPI-I PCR product. B) Avidin matu
30 protein cDNA fragment, resulting from a BamH I/Hind III digest of the mutated Avid
PCR product. C) pART7 vector following an Xho I/Hind III digestion. * denot
compatible cohesive ends. ** denotes compatible cohesive ends.

Figure 6 shows DNA fragments A, B and C were the components of the ligation reacti
35 to produce recombinant pUC19 containing the PPI-II signal sequence/Streptavidin cDN
gene fusion. The fused gene was then released from pUC19 by a Sal I/BamH I digest a
ligation of components D and E produced recombinant pART7. A) PPI-II leader fragme

resulting from a Sal I/EcoR I digest of the PPI-II PCR product. B) Streptavidin cDNA fragment, resulting from an EcoR I/Xba I digest of the recombinant plasmid pUC19/Streptavidin cDNA. D) PPI-II signal sequence/Streptavidin cDNA gene fusion fragment, resulting from a Sal/BamH I digest of recombinant pUC19 containing the fused gene. E) pART7 vector following an Xho I/BamH digestion. * denotes compatible cohesive ends.

Figure 7 shows a schematic representation of the pART7 expression cassette as it was cloned into the pART27 binary vector; A) containing the PPI-I-Avidin gene fusion and B) containing the PPI-II/Streptavidin gene fusion.

Figure 8 shows PPI-I/Avidin gene fusion sequence (A) and fusion protein sequence (B): The fusion protein has a total of 161 amino acids; the PPI-I sequence is represented by italic type with bold type denoting the PPI-I signal peptide. Two amino acids, novel to both the PPI-I and the Avidin peptide sequences and represented in lower case were introduced with the ligation of the Bgl II and BamH I compatible cohesive ends.

Figure 9 shows PPI-II/Streptavidin gene fusion sequence (A) and fusion protein sequence (B): The fusion protein has a total of 168 amino acids; the PPI-II sequence is represented by italic type with bold type denoting the PPI-II signal peptide. Three amino acids, novel to both PPI-II and the Streptavidin peptide sequences and represented in lower case were introduced at the point of fusion.

Figures 10 and 11 show the mortality of potato tuber moth larvae on whole tobacco plants expressing the avidin gene in two replicate trials.

Figure 12 (A) shows nucleotide sequence for the gene for streptavidin (Argarana C.E. et al. 1986). The signal sequence is represented in bold type, start and stop codons in bold italic. (B) shows protein sequence for streptavidin the signal sequence is represented in bold type.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel chimeric polypeptides comprising vacuole targeting sequences and biotin-binding sequences. The targeting sequences and biotin binding sequences are operably linked.

The term "operably linked" as used herein refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For example, a signal sequence is operably linked to a coding sequence if the promoter affects its transcription or expression.

5 The vacuolar targeting polypeptide sequences of the invention, when transformed into plants, function to direct the protein products directed by the expression of genes to which they are operably linked from the cytoplasm to the vacuole of the plant cell. Since the vacuole of plant cells has a storage function, proteins directed there remain there,
10 continually increasing in abundance, unless subject to degradation by vacuolar proteinases. The vacuolar proteins are also isolated from the major metabolic processes in the plant and thus will not interfere with the plant growth and development. The success of the present invention required that both these requirements be met.

15 Vacuolar targeting sequences include any such targeting sequences as are known in the art. These include polypeptides targeting barley lectin (Bednarek et al., 1990)³, sweet potato sporamin (Matsuoka et al., 1990)¹⁶, tobacco chitinase (Neuhaus et al., 1991)²³, bean phytohemagglutinin (Tague et al., 1990)²⁸, 2S albumin (Saalbach G et al., 1996)²⁴,
20 aleurain (Holwerda et al., 1992)¹⁴. See also references 7, 8, 13, 25, 33, 32 and 35-46 referenced on pages 27 to 31. However, potato proteinase inhibitor targeting sequences are preferred.

A number of potato proteinase signal sequence polypeptides designated PPI-I and PPI-II are disclosed herein. The polypeptides have the amino acid sequences set out in Figures
25 8b and 9b respectively. Also encompassed within the invention are variants of these polypeptides and those known in the art which have substantially equivalent targeting sequence activity thereto.

The term "variant" as used herein refers to a polypeptide wherein the amino acid sequence
30 exhibits substantially 70% or greater homology with the amino acid sequences set out in Figures 1 and 4. Preferably, the variants will have greater than 85% homology, and most preferably, 95% homology or more. Variants may be arrived at by modification of the native amino acid sequence by such modifications as insertion, substitution or deletion of one or more amino acids.

35

As noted above, chimeric polypeptide comprising a vacuolar targeting signal sequence operably linked to a biotin-binding protein. Biotin is an essential nutrient for many

species of pests (Dadd, R.H., (1985)⁹. Nutrition: Organisms In: Comprehensive In Physiology, Biochemistry and Pharmacology (Kerkut G.A. and Gilbert, L.L.) Pergamon Press, NY Vol 4, p313-390). As discussed above, biotin-binding proteins have been found to have pesticidal properties and to inhibit growth of pests. The binding of biotin causes a biotin deficiency which results in the inhibition of growth and ultimate death of pests.

The term "pest" as used herein refers to a broad group of organisms which have a biotin requirement, including protozoa, arthropods (especially insects), aschelminths, platyhelminthes, nematodes and molluscs.

Biotin-binding proteins known in the art include egg yolk (Subramanian & Adiga, 1990) serum (Seshagiri & Adiga, 1987)²⁶, and bacterial proteins, avidin, isolated from egg white and streptavidin.

Preferred biotin-binding polypeptides, for use in the present invention, are avidin, streptavidin or functionally equivalent variants thereof. It will be appreciated that other proteins that function to bind biotin are equally able to be used in the present invention.

Avidin is a water-soluble tetrameric glycoprotein isolated originally from raw egg white (J. Biol.Chem 136: 801 (1940)). The protein is well known with the complete amino acid sequence having been published in, for example, J.Biol.Chem. 246: 698 (1971). The amino acid sequence for avidin is shown in Figure 8b (amino acids 34 to 161).

Streptavidin is a non-glycosylated bacterial binding protein derived from the culture supernatant of *Streptomyces avidinii* (Bayer, E.A., et al., 1990)². The full amino acid sequence for streptavidin is given in Figure 12.

'Core' SAV is equivalent to amino acid residues 37-164 of *Streptomyces avidinii* (See Figure 12, (Argarana et al., (1986))¹. A preferred sequence referred to as "Synthetic 'Core' Streptavidin" is a modified 'core' SAV having the sequence shown in Figure 9b (amino acids 41 to 168). SYNSAV is equivalent to 'Core' SAV modified such that codons for each amino acid correspond to those in highly expressed E.coli genes. SYNSAV is modified to contain unique restriction sites evenly throughout sequence. The resulting sequence has G + C content of 54% relative to 69% for same region of native SAV (Thompson, L.D., et al. (1993))²⁹.

The reader will appreciate that modifications, including chemical and biochemical

modifications, of the polypeptides of the invention are possible. Such modifications include, for example, acetylation, carboxylation, phosphorylation, glycosylation, ubiquitination, labeling, and the like. The production of peptide fragments is also well within the capabilities of an art skilled worker.

5

The polypeptides of the invention can be prepared in a variety of ways. For example, as indicated above for the signal sequences and biotin-binding proteins can be produced by isolation from natural sources and then coupled using techniques known in the art. For example, through recombinant nucleic acid methods.

10

Synthesis using known techniques (such as stepwise solid phase synthesis described by Merryfield (1963), *J. Amer.Chem.Soc.* Vol 85:2149-2156), or as preferred through employing recombinant DNA techniques.

15 The variants of both the polypeptide and peptides can similarly be made by any of those techniques known in the art. For example, variants can be prepared by site-specific mutagenesis of the DNA encoding the native amino acid sequence as described by Adelman et al. *DNA* 2:183 (1983).

20 Where it is preferred, recombinant techniques used to produce the polypeptide or peptide of the invention, the first step is to obtain DNA encoding the desired product. Such DNA comprises a still further aspect of this invention.

25 The DNA of the invention may encode a native or modified polypeptide or peptide of the invention or an active fragment thereof. In its presently preferred forms, the DNA comprises the nucleotide sequence of Figure 8A, or the nucleotide sequence of Figure 9A. Preferred sequences exhibit 60% or greater homology with these sequences, preferably 80% homology and most preferably 95% homology or more. That is, most preferred sequences will hybridise to the sequences of the invention under stringent hybridisation
30 conditions.

The DNA can be isolated from any appropriate natural source or can be produced as intron free cDNA using conventional techniques. DNA can also be produced in the form of synthetic oligonucleotides where the size of the active fragment to be produced permits.
35 By way of example, the Triester method of Matteucci et al. *J. Am.Chem.Soc.* Vol 103:3185-3191 (1981) may be employed.

Where desirable, the DNA of the invention can also code for the chimeric polypeptide of the invention. Fusion proteins further comprising the polypeptide or peptide of the invention and a carrier protein are possible. This carrier protein will generally be cleavable from the polypeptide, peptide or fragment under controlled conditions. Examples of commonly employed carrier proteins are β -galactosidase and glutathione transferase.

As indicated above, also possible are variants of the polypeptide or peptide which differ from the native amino acid sequence by insertion, substitution or deletion of one or more amino acids. Where such a variant is desired, the nucleotide sequence of the native DNA is altered appropriately. This alteration can be made through elective synthesis of DNA or by modification of the native DNA by, for example, site-specific or cassette mutagenesis. Preferably, where portions of cDNA or genomic DNA require sequence modifications, site-specific primer directed mutagenesis is employed using techniques standard in the art.

In a further aspect, the present invention consists in replicable transfer vectors suitable for use in preparing a polypeptide or peptide of the invention. These vectors may be constructed according to techniques well known in the art, or may be selected from among the cloning vectors available in the art.

The cloning vector may be selected according to the host or host cell to be used. Use of the vectors will generally have the following characteristics:

- (a) the ability to self-replicate;
- (b) the possession of a single target for any particular restriction endonuclease; and
- (c) desirably, carry genes for a readily selectable marker such as antibiotic resistance or herbicide tolerance.

Two major types of vector possessing these characteristics are plasmids and bacteriophages (bacteriophages or phages). Presently preferred vectors include the plasmids pMOS-Blue, pGem-T, pUC18, pUC19, pART27, pMON, pJIT, pBIN, pRD 400, pART1

The DNA molecules of the invention may be expressed by placing them in operable linkage with suitable control sequences in a replicable expression vector. Control sequences may include origins of replication, a promoter, enhancer and transcription terminator sequences amongst others. The selection of the control sequence to

included in the expression vector is dependent on the type of host or host cell intended be used for expressing the DNA.

Generally, procaryotic, yeast, insect or mammalian cells are useful hosts. Also include
5 within the term hosts are plasmid vectors. Suitable procaryotic hosts include *E. coli*
Bacillus species and various species of *Pseudomonas*. Commonly used promoters such
as β -lactamase (penicillinase) and lactose (lac) promoter systems are all well known in
the art. Any available promoter system compatible with the host of choice can be used.
Vectors used in yeast are also available and well known. A suitable example is the
10 micron origin of replication plasmid.

Similarly, vectors for use in mammalian cells are also well known. Such vectors include
well known derivatives of SV-40, adenovirus, retrovirus-derived DNA sequences, Herpes
simplex viruses, and vectors derived from a combination of plasmid and phage DNA.
15

Further eucaryotic expression vectors are known in the art (e.g. P.J. Southern and P. Berg,
J. Mol. Appl. Genet. 1 327-341 (1982); S. Subramani et al., *Mol. Cell. Biol.* 1, 854-864
(1981); R. J. Kaufmann and P.A. Sharp, "Amplification and Expression of Sequences
Cotransfected with a Modular Dihydrofolate Reducase Complementary DNA Gene, *J.*
20 *Mol. Biol.* 159, 601-621 (1982); R. J. Kaufmann and P.A. Sharp, *Mol. Cell. Biol.* 159, 601-
664 (1982); S.I. Scahill et al., "Expressions And Characterization Of The Product Of A
Human Immune Interferon DNA Gene In Chinese Hamster Ovary Cells," *Proc. Natl.*
Acad. Sci. USA. 80, 4654-4659 (1983); G. Urlaub and L.A. Chasin, *Proc. Natl. Acad. Sci.*
USA. 77, 4216-4220, (1980).
25

The expression vectors useful in the present invention contain at least one expression
control sequence that is operatively linked to the DNA sequence or fragment to be
expressed. The control sequence is inserted in the vector in order to control and to
regulate the expression of the cloned DNA sequence. Examples of useful expression
30 control sequences are the lac system, the trp system, the tac system, the trc system, major
operator and promoter regions of phage lambda, the glycolytic promoters of yeast acid
phosphatase, e.g. Pho5, the promoters of the yeast alpha-mating factors, and promoters
derived from polyoma, adenovirus, retrovirus, and simian virus, e.g. the early and late
promoters of SV40, and other sequences known to control the expression of genes of
35 prokaryotic and eucaryotic cells and their viruses or combinations thereof.

Preferred promoters for use herein include lacZ, CaMV-35S, LHC a/b, T7, nos, rubisco

small subunit (SSU), *gpd* and *nod* gene promoters.

In the construction of a vector it is also an advantage to be able to distinguish the vectors incorporating the foreign DNA from unmodified vectors by a convenient and rapid assay.

5 Such assays include measurable colour changes, antibiotic resistance, herbicide tolerance and the like. In one preferred vector, the β -galactosidase gene is used, which gives a readily detectable blue phenotype on X-gal plates. This facilitates selection.

10 Once selected, the vectors may be isolated from the culture using routine procedures such as freeze-thaw extraction followed by purification.

For expression, vectors containing the DNA of the invention to be expressed and control signals are inserted or transformed into a host or host cell. Intermediate host cells can be used to increase the copy number of the cloning vector prior to introduction into production cells. Some useful expression host cells include well-known prokaryotic and eucaryotic cells. Some suitable prokaryotic hosts include, for example, *E. coli*, such as *E. coli*, S 936, *E. coli* HB 101, *E. coli* W3110, *E. coli* X1776, *E. coli*, X2282, *E. coli*, DHT, and *E. coli*, MR01, *Pseudomonas*, *Bacillus*, such as *Bacillus subtilis*, and *Streptomyces*. Suitable eucaryotic cells include yeast and other fungi, insect, animal cells, such as COS cells, CHO cells, human cells and plant cells in tissue culture.

Expression systems employing insect cells utilising the control systems provided by baculovirus vectors have been described (Miller, D W et al., in *Genetic Engineering* 25 (1986) Setlow W, J K et al., Eds, Plenum Publishing, Vol 8, pages 277-297).

Depending on the host used, transformation is performed according to standard techniques appropriate to such cells. For prokaryotes or other cells that contain substantial cell walls, the calcium treatment process (Cohen, S N *Proceedings, National Academy of Sciences USA* 69 2110 (1972)) may be employed. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graeme and Van Der Eb, *Virology* 52: 1 (1978) is preferred. Transformations into plants may be carried out using *Agrobacterium tumefaciens* (Shaw et al., *Gene* 23:315 (1983) or into yeast according to the method of Van Solingen et al. *J. Bact.* 130: 946 (1977) and Hsiao et al. *Proceedings, National Academy of Science*, 76: 3829 (1979). 35

In a preferred transformation process, the vectors of the invention are incorporated into

Agrobacterium tumefaciens which can be used to infect plant cells, particularly dicotyledonous plant cells, thereby transferring the vectors and conferring pest resistance. The cloning vectors can also be introduced into plant cells using convenient art techniques such as electroporation, microparticle bombardment and microinjection. Microparticle bombardment is the preferred transformation process for monocotyledonous plants. Suitable plant transformation techniques are usefully summarised in Torres et al., Cell, Tissue and Organ Culture 34: 279-285 (1993), Michelmore et al., Plant Cell Reports 6:439-442 (1987), Horsch et al., Plant Molecular Biology Manual AS: 1-9 (1988), Xu et al., J. Genet. and Breed. 46: 287-290 (1992) and WO 97/17455 incorporated here by reference.

Upon transformation of the selected host with an appropriate vector the polypeptide encoded can be produced, often in the form of fusion protein, by culturing the host. The polypeptide of the invention may be detected by rapid assays as indicated above. The polypeptide can then be recovered and purified if desired. Recovery and purification may be achieved using any of those procedures known in the art, for example by absorption onto and elution from an anion exchange resin. This method of producing a polypeptide of the invention constitutes a further aspect of the present invention.

The present invention also provides a method for producing avidin or streptavidin by a method comprising extracting avidin or streptavidin from a plant incorporating a DNA sequence of the invention coding for avidin or streptavidin. The expression level of avidin or streptavidin may be increased by further incorporating into the DNA sequence of the invention a peptide export signal sequence, or intron sequence. Methods of enhancing expression levels and method for production of avidin and streptavidin generally may be effected according to the techniques of WO 97/17455 incorporated herein by reference. The use of the chimeric polypeptides of the present invention represents an advance in this document because the avidin or streptavidin is produced in vegetative tissues (leaves, stems, tubers, roots) as opposed to the reproductive tissues. The method can be used to produce avidin or streptavidin from a wide range of plants which produce abundant vegetative material e.g. potatoes, cassava, tobacco, grasses, legumes, and trees rather than be restricted to plants which produce large reproductive structures e.g. maize.

Plants suitable for transformation with the vectors of the invention may be selected from a broad range of plants including cereal crops, vegetable, fruit and other food crops, fibre crops and turf plants, fibre crops, timber and pulp and paper plants, shelter-belt plants, tree crops, ornamental and flower plants, culinary plants, medicinal plants and herbs.

plants grown to produce beverages.

Examples of cereal crops include wheat, rice, barley, maize, oats, millet, sorghum and

5 Examples of vegetable, fruit and other food crops include root crops such as potato, s
potato, beetroot, parsnip, turnip, swede and carrot, cucurbits such as cucum
pumpkins, squash, marrow, courgettes and watermelon, brassicas such as cauliflo
cabbage, oilseed rape, brussels sprouts and broccoli, corn, tomato, lettuce, celery, oni
garlic, legumes such as lentils, green beans, lima beans, haricot beans, red kidney be
10 kudzu beans, mung beans, broadbeans, soybeans, chickpeas, peas, and peanuts, a
pear, kiwifruit, tamarillo, feijoa apricot, plum, citrus such as orange, lemon, tang
grapefruit, uglifruit and mandarin, pineapple, peach, nectarine, cherry, berries, olives
sugarcane.

15 Examples of forage crops and turf plants include legumes such as clover, alfalfa, l
trefoil and lucerne and grasses and other graminaceous plants such as ryegrass, brow
fescue, cocksfoot, kikuyu and, paspalum, and sorghum grass.

Fibre crops include cotton, flax, kapok and hemp. Timber, shelterbelt, conservation,
20 and paper plants and tree crops include, for example, pine, eucalyptus, spruce, fir,
ash, birch, beech, mahogany, rosewood, ebony, maple, teak, cedar, redwood, ja
chestnut, walnut, macadamia nut, poplar, willow, cypress, camphor, mulberry, mai
grass and rubberplant.

25 Ornamental shrubs, trees and flower plants include roses, petunias, orchids, carnati
chrysanthemums, daisies, tulips, lilies, gypsophylla, hibiscus, rhododendrons, con
camellias, hebes, lavender, lupins, tussock, ferns and native plants. Culinary pl
include herbs such as basil, rosemary, oregano, bay, and spices such as cinnamon, m
tumeric, and sage.

30 Medicinal plants include poroporo, opium poppies, coca, marijuana, camomile, com
foxglove, belladonna.

Plants used to produce beverages include tea, coffee, hops and cocoa.

35 Plants transformed with the vectors of the invention direct expression of the biotin bin
proteins in the vacuoles of the plant cells. The biotin binding protein is effecti

sequestered into the vacuole. When an insect pest feeds on the plant, the plant cell components mix together allowing biotin to be bound by the binding protein. This essentially deprives the insect of the vitamin it requires leading to stunted growth and death.

5

The present invention has application in producing plants resistant to a broad range of pests in adult or larval stage including moths, beetles, weevils, caterpillars, borers, budworms, armyworms, bollworms, rootworms, webworms, aphids, bugs, crickets, locusts, grubs, flies, fruitflies, leafminers, plant hoppers, earwigs, scale insects, thrips, and
10 springtails. Plants of the invention may also be resistant to other invertebrate pests of plants such as slugs, snails, mites, lice and nematodes and other pests and pathogens which have a vitamin requirement for biotin.

Accordingly, in a further aspect the invention provides a method of imparting pest
15 resistance to plants comprising transforming the plants with a vector according to the present invention.

The method may also be effected by transforming isolated plant cells or tissues and generating plants from the transformed cells or tissue using standard culture techniques.
20 Plant cells and tissue cultures transformed with vectors of the invention form further aspects of the invention.

Transformed plants can be used in conventional breeding programmes to transfer the DNA sequences of the invention.

25

In another aspect, the present invention also provides a composition comprising a chimeric polypeptide of the invention and a carrier diluent, excipient or adjuvant therefor.

Preferably, the composition is a pesticidal composition comprising a pesticidally effective
30 amount of the polypeptide and an acceptable carrier. The pesticidal composition can be applied to plants in the form of sprays, dusts, or other formulations commonly employed in making pesticides.

In another embodiment, the composition may be applied to harvested material to prevent
35 pest damage in storage. In an extrapolated application, the compositions may similarly be used in plant derived products such as flours, meals, cereals and the like to prevent or control pest infestation.

It will be appreciated that the above description is provided by way of example only and that variations in both the materials and techniques used which are known to those persons skilled in the art are contemplated.

- 5 Non-limiting examples illustrating the invention will now be provided.

EXAMPLE 1

Materials

Custom primers were synthesized by Life Technologies. Subcloning Efficiency DH5
10 competent Cells were purchased from Life Technologies and the Hybaid Recovery
Plasmid Mini Prep Kit from Hybaid Limited. All enzymes, unless otherwise stated were
purchased from Promega. Ampligase Thermostable DNA Ligase and Reaction Buffer and
GELase were purchased from Epicentre Technologies and Polymerase Chain Reaction
(PCR) reagents from Perkin Elmer.

15

The Avidin cDNA (pGEMav) carried on the plasmid pGEM3 was supplied by Professor
M. S. Kulomaa ((Department of Biological and Environmental Science, University of
Jyvaskyla, Finland) and the Potato Proteinase Inhibitor I (PPI-I) cDNA was isolated in this
laboratory (Beuning et al. 1994, GenBank Accession # L06606) and cloned into pUC19.

20

The Streptavidin cDNA, carried on the plasmid pET3a was supplied by The DuPont
Merck Pharmaceutical Company. The Potato Proteinase Inhibitor II (PPI-II) genomic
sequence was isolated in this laboratory and cloned into pUC19 (Murray C. and
Christeller J. T. 1994)²¹.

25

Methods

Subcloning Efficiency DH5 competent Cells were used for general cloning and
amplification of recombinant plasmids and the Hybaid Recovery Plasmid Mini Prep Kit
was used for plasmid preps. Isolation and recovery of DNA fragments was achieved by
30 agarose gel electrophoresis followed by treatment of excised gel bands with GELase.

DNA Sequencing and Computer Analysis:

DNA sequencing was carried out on an Applied Biosystems (ABI) DNA Sequencer using
35 dye terminator chemistry. Sequence analysis was performed using the Wisconsin Package
Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin.

EXAMPLE 2

Preparation of a binary vector designed to express a chimeric polypeptide comprising Avidin mature peptide fused to a Potato Proteinase Inhibitor I Signal Peptide

5

Methods

A one-step PCR-based mutagenesis method employing the combined use of thermostable DNA polymerase and thermostable DNA ligase (Moore D.S. and Mich S. F. 1995)¹⁹, was used to prepare a construct comprising the sequence encoding mature Avidin polypeptide (Gope M.L. et al. 1987)¹² fused to a PPI-I signal sequence. A Bgl II site was produced downstream of the PPI-I leader sequence at positions 92 - 97 of the PPI-I coding sequence and a BamH I site was created upstream of the sequence encoding the mature Avidin polypeptide, at positions 65 - 70 of the sequence encoding Avidin protein, as shown in Fig. 1 and Fig. 2 respectively. These two restriction sites have compatible cohesive ends.

15

Primers:

Forward M13 (lacZ) Primer [Perkin Elmer]:

20 5'-GCCAGGGTTTCCAGTCACGA-3'

Reverse M13 (lacZ) Primer [Perkin Elmer]:

5'-GAGCGGATAACAATTTACACAGG-3'

25

Avidin Upstream Primer:

5'-GCACACCCGGCTGTCCACCTG-3'

30 Phosphorylated Mutagenic Primers

PPI-I mutagenic primer:

5'-PGATGGACCAGAGATCTTAGAAC-3'

35

Avidin mutagenic primer:

5'-PGGCTCCCGGGATCCCTGCCAG-3'

Amplification/Mutagenesis reactions

To generate mutant products a total PCR reaction volume of 50 ul with an effective

- 5 Ampligase Reaction Buffer [20 mM Tris-HCl (pH 8.3 at 25°C), 25 mM KCl, 10 mM MgCl₂, 0.5 mM NAD and 0.01% Triton X-100] was used with the following conditions:

- 100 pmol each outer primer
1 nmol phosphorylated mutagenic primer
10 40 nmol each dNTP
0.1 umol dithiothreitol
5 U Taq DNA polymerase
5 U thermostable DNA ligase
1 ng recombinant plasmid DNA template

15

Reactions were first incubated at 94°C for 3 min., followed by 30 amplification cycles performed as follows:

- 94°C, 1 min.
20 40°C, 1 min.
65°C, 6 min.

Amplification cycles were followed by a final extension at 65°C for 7 min.

- 25 Restriction analysis of amplification products from both mutagenesis reactions revealed mutant product to be present, but only at a maximum of 5% of the total product. To increase the yield of mutated product, Bgl II (for PPI-I mutagenesis) and BamH I (for Avidin mutagenesis) digestion products were ligated and then used as template for second amplification reaction using outer primers only (Avidin Upstream and Reverse
30 M13 (lacZ) for AVIDIN; Forward M13 (lacZ) and Reverse M13 (lacZ) for PPI-1). For PPI-I, greater than 95% of second round amplification product had the desired Bgl II site and approximately 80% of the second round product for Avidin mutagenesis possessed the BamH I site.
- 35 The mutated PPI-I amplification product was digested with Bgl II and Sal I and the mutated Avidin product with BamH I and Hind III. The PPI-I leader sequence and coding sequence for the Avidin mature protein were isolated and recovered for cloning.

along with Xho I/Hind III digested non-recombinant pART7 vector (Gleave A.P.1992)¹. These three species were ligated, resulting in recombinant pART 7 [refer Fig. 5] and the sequence of the chimeric gene was checked. Subsequently, the expression cartridge containing the gene fusion was cloned into the Not I site of pART27 vector (Gleave A.P.1992)¹¹ and this construct [refer Fig. 7A] was mobilized to *Agrobacterium tumefaciens* (strain LBA4404) by standard tri-parental mating techniques (Ditta G. et al 1980)¹⁰.

Discussion

- 10 The resulting PPI-I/Avidin fusion protein has a total of 161 amino acids as shown in Fig. 8. The first 31 amino acids are PPI-I sequence and since the leader sequence comprises the first 23 amino acids, the original patterning of amino acids around with the site for cleavage between the signal sequence and the mature protein is retained. There are two single base pair changes in the gene fusion sequence relative to the predicted sequence.
- 15 These changes are presumably the result of PCR error. One change is silent and the other results in an amino acid change from Serine to Proline at position 17 of the PPI-I signal sequence.

EXAMPLE 3

Preparation of a binary vector designed to express a chimeric polypeptide comprising Synthetic "Core" Streptavidin peptide fused to a Potato Proteinase Inhibitor II Signal Peptide

Methods

- 25 A fused gene was prepared comprising the sequence encoding Synthetic "Core" Streptavidin (Thompson L. D. and Weber P. C. 1993)²⁹ fused to a PPI-II signal sequence. The Streptavidin cDNA, carried on the plasmid pET3a was cloned into the EcoR I/Xba I sites of pUC 19 (Fig. 3). The PPI-II signal sequence (Fig. 4) which contains an intron was isolated from recombinant plasmid using PCR with a sense primer binding to pUC19
- 30 and an antisense primer incorporating an EcoR I site into a 5' overhang. The primers were as follows:

sense primer:

35 5' - CTG CAG GTC GAC TCT AGA GGA - 3'

antisense primer:

5' - GGT GAA TTC TTA GTA CAG ATC TTC GCA - 3'

Amplification reaction

A total PCR reaction volume of 50 ul with an effective 1 X PCR Buffer [10 mM Tris-H
5 pH 8.3 and 50 mM KCL] was used with the following conditions:

20 pmol each primer
15 nmol each dNTP
2.0 mM MgCl₂
10 5 U Taq DNA polymerase
1ng recombinant plasmid DNA template

Reactions were first incubated at 94°C for 2 min., followed by 30 amplification cycles
performed as follows:

15 94°C, 1 min.
50°C, 1 min.
72°C, 1 min.

Amplification cycles were followed by a final extension at 72°C for 7 min.

20 The PCR product representing the PPI-II signal sequence was digested with Sal I and
EcoR I. The recombinant plasmid pUC 19/Streptavidin cDNA was digested with EcoR
I and Xba I and the Streptavidin cDNA was isolated from the vector and recovered. Next
recombinant pUC19 was digested with Sal I and Xba I and the three species were ligated
25 to produce a construct comprising the gene fusion cloned into the Sal I and Xba I sites
of pUC19. The sequence of the gene fusion was checked and subsequently cloned into the
Xho I and BamH I sites of the pART7 vector [refer Fig. 6]. The pART7 expression
cartridge containing the gene fusion was then cloned into the Not I site of pART27 and
this construct [refer Fig. 7B] was mobilized to *Agrobacterium tumefaciens* (strain
30 LBA4404) by standard tri-parental mating techniques.

Discussion

The resulting PPI-II/Streptavidin fusion protein has a total of 168 amino acids as shown
in Fig. 9. The first 36 amino acids are PPI-II sequence. Five of these amino acids follow
35 the cleavage site, preserving the amino acid pattern around this position. The nucleotide
sequence of the PPI-II signal sequence includes a 119 bp intron (Murray C. and Christell
J. T. 1994)²¹.

EXAMPLE 4

Immunodetection of avidin in transgenic tobacco

Methods

5

1. Tissue print

Pieces of transgenic tobacco leaves 1 x 1cm were frozen at -20 C for 20 min, allowed to thaw and printed on to nitrocellulose using mechanical pressure. The printed nitrocellulose membrane was washed in PBS-T (phosphate buffered saline with 0.1% Tween 20) for 20
10 min, blocked in 0.1% BSA-C (Aurion) for 15min and incubated in 1:1000 anti-avidin (Sigma A-5170) diluted in blocking buffer for 1h (This last step is deleted for control). The membrane was then washed in PBS-T, incubated in goat anti-rabbit IgG-gold (10nm) (Sigma), washed again in PBS-T, then in ddH₂O and drained. Finally the membrane was silver enhanced (BioCell silver enhancement kit) for 15min.

15

2. Embedded material

Pieces 1 x 1x 5mm of transgenic tobacco leaf were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer under vacuum for 1h. The material was post-fixed in 1% osmium tetroxide 1h, dehydrated in an ethanol series and embedded in
20 Spurr's resin. Sections were cut 1µm thick for light microscopy (LM) and mounted on Poly-L-lysine coated slides. Sections for electron microscopy (EM) were cut 130nm thick (gold) and mounted on carbon/formvar coated nickel grids.

For light microscopy the sections had a Pap pen ring drawn around them to contain the
25 incubation liquid. The protocol for LM and EM were the same thereafter. The sections were etched for 30min in 10% hydrogen peroxide to remove the osmium, blocked in 0.1% BSA-c for 15 min, incubated in anti-avidin 1:100 in PBS-T for 1h and washed in PBS-T. They were then incubated in goat anti-rabbit IgG-gold (10nm) for 1h, washed in buffer, then water and finally silver enhanced for 7 min for the LM section and counter
30 stained with toluidine blue.

Results

The nitrocellulose membrane silver enhanced (turned brown) over the entire area of the tissue print. There was no silver enhancement on the control print. This labelling protocol
35 also acts as a test of the labelling procedure.

Immunolabelling of LM and EM sections showed labelling of protein-type bodies in the

vacuoles of mesophyll cells (both spongy and palisade) and in glandular hairs. protein bodies were usually condensed into one body which was sometimes seen as a r There was a lower level of labelling in the cytoplasm.

5 Conclusions

The results indicate that avidin is synthesized in most common cell types in tobacco leaves. The bulk of the protein appears to be transported to the vacuole and deposited a protein body within this organelle.

10

EXAMPLE 5

Feeding trials with neonate potato tuber moth larvae on whole tobacco plants expressing avidin

15 Constructs:

Non-transformed control plants

- 2 plants (NT 1, NT 2)

Control plants transformed with pumpkin fruit chymotrypsin inhibitor (PFCI) but not expressing the protein

20 - 3 plants (JB3/1, J13312, JB5/1)

Tobacco plants transformed with the avidin gene with a PPI-1 targeting sequence (example 2 above)

- 6 lines (PLA2/2, PLA2/7, PLA2/9, PLA2/13, PLA2/20, PLA2/24), 4 clones per line.

25

Trial Design

Trial i

The tobacco plants were removed from tissue culture and potted in fertilised potting mix (Smiths® general potting mix) before being placed in large ventilated acetate containers (220 x 300mm) in a containment glasshouse unit at 22±5°C. They were watered daily to maintain high humidity and soil moisture content.

35 Eight days later, when plants were well established with at least 4-5 small leaves, tobacco neonate potato tuber moth (PTM) larvae were placed on each tobacco plant, usually two per leaf. Prior to inoculation the larvae were weighed in batches of five (since single larvae are too small to give an accurate reading). TM larvae were obtained from

laboratory culture reared on potato tubers following the same basic procedure as Brooker (1971)⁶ and Meisner et al. (1974)¹⁷.

Trial ii

One week after Trial i was completed, the tobacco plants were cut back to the second node and allowed to regenerate leaves. When the plants had developed 4-5 leaves (approximately 11d) they were each inoculated again with ten neonate PTM larvae, usually 2 per leaf, weighed in batches of five prior to inoculation as above.

Trials i and ii

Inoculated plants were kept individually in acetate containers in the containment glasshouse unit at 22 ± 5 C for nine days. Under these conditions growth of control larvae is exponential from hatch to nine days, but after this growth rate slows as pupation approaches. Hence in order to compare growth rates of larvae on control and transgenic plants, the trial was concluded after nine days. Damaged leaves containing larvae were removed and photographed, and larvae were dissected out of their mines within the leaf or stem tissue. The intention was to weigh the larvae at this point in order to estimate growth rates, but, except for those on control plants, larvae were mostly dead, dried and shrivelled. Consequently head capsules were measured so that the instar reached at death could be recorded.

Results

Level of expression of the avidin protein

The level of expression of avidin in each of the plant lines was quantitated using chemiluminescence and expressed as percentage of total leaf protein. These levels are given in Table 1 below.

Table 1 The level of expression of avidin as % of total leaf protein, determined using the chemiluminescence method

5	Plant Line	Level of expression of avidin (% total leaf protein)
	PLA2/2	0.07
10	PLA2/7	0.10
	PLA2/9	0.07
	PLA2/13	0.06
15	PLA2/20	0.065
	PLA2/24	0.06

20 Mortality of PTM Larvae feeding on whole tobacco plants expressing the avidin gene

Trial i

Good recovery rates of larvae from both control and transgenic plants were obtained: 86% from controls and 76.7% from transformed plants. Fig. 10 clearly shows the high mortality

25 PTM larvae after feeding for nine days on whole transgenic tobacco plants expressing the avidin gene compared to both non-transformed control plants and control plants transformed with, but not expressing, the pumpkin fruit chymotrypsin inhibitor (PFCI) gene.

30 The majority of dead larvae were recovered from mines where they had died at the "cutting face". A few (5% of dead larvae) were recovered from the surface of leaves, having generally left a mine close by. It is most likely that the majority of larvae not recovered had died in this way and had fallen off the leaves. Some mines were found without occupants. However, there was no evidence that larvae had started and abandoned
35 mines.

PTM larvae undergo four instars during their development. In order to define the stage of development of the larvae at death, head capsule widths were measured using a micrometer eye-piece. All control larvae were alive and most were third instars. None of
40 the larvae recovered on any of the plants expressing avidin had reached third instar before death and many had died during or just after the moult from first to second instar, as

evidenced by the fact that the ecdysed cuticle was still attached. This reflected results in earlier trials with avidin incorporated into diet. Table 2 below gives a breakdown of instars on each plant line.

5 **Table 2** Number of larvae at each instar recovered from transgenic tobacco plants expressing avidin in Trial i

	Plant line	Neonates inoculated	Number of larvae at			
			1st instar	2nd instar	3rd instar	4th instar
10	NT control	20	0	1	18	0
	JB3 control	30	0	0	23	1
	PLA2/2	40	3	28	0	0
	PLA2/7	40	4	23	0	0
	PLA2/9	40	2	27	0	0
15	PLA2/13	40	1	25	0	0
	PLA2/20	40	2	27	0	0
	PLA2/24	40	4	25	0	0

Trial ii

- 20 Again there were good recovery rates of larvae from both control and transgenic plants: 88% from controls and 88.8% from transformed plants. Fig. 11 clearly reflects the results of the first trial showing high mortality of PTM larvae fed on whole transgenic tobacco plants expressing the avidin gene compared to those on control plants. In fact a total of only 4 live larvae were recovered from all avidin-expressing plants (<1.7% survival),
 25 whereas only 3 larvae had died on the control plants (94% survival).

Head capsule widths of larvae were measured and the number of recovered larvae at each instar is given in Table 3. As in the first trial, none of the larvae recovered from any of the plants expressing avidin had reached third instar before death and many had died
 30 during or just after the moult from first to second instar; again the ecdysed cuticle was still attached in several cases.

Table 3 Number of larvae at each instar recovered from transgenic tobacco plants expressing avidin in Trial ii

5	Plant line	Neonates	Number of larvae at			
		inoculated	1st instar	2nd instar	3rd instar	4th instar
	NT control	20	0	3	12	
	JB3 control	30	0	1	24	
	PLA2/2	40	34	5	0	
	PLA2/7	40	25	9	0	
10	PLA2/9	40	30	3	0	
	PLA2/13	40	26	11	0	
	PLA2/20	40	25	5	0	
	PLA2/24	40	30	3	0	

15 **Conclusion**

Total mortality of PTM larvae fed on tobacco plants expressing the avidin gene was 100%. No significant mortality have occurred if the trials had been continued beyond nine days; larvae that survived nine days were small, shrivelled and close to death as evidenced by their minimal response when touched by a paintfine sable brush.

20

Avidin expressed in tobacco plants is highly toxic to PTM larvae and has definite potential in the development of pest resistant crop cultivars.

REFERENCES

- 1) Argarana C.E., Kuntz, I.D., Birkens, Axel R., and Cantor C.R. (1986). Molecular Cloning and Nucleotide Sequence of the Streptavidin Gene Nucleic Acids research
5 14: 1871-1882.
- 2) Bayer, E.A., H. Ben-Hur, and M. Wilchek, 1990, Isolation and Properties of Streptavidin, In: Avidin-biotin Technology, Met. Enzymol. Vol 101 (Wilchek, M. and Bayer, E.A., eds) Academic Press, New York, pp 80-89.
10
- 3) Bednarek SY, Wilkins TA, Dombrowski JE, Raikhel NV (1990) A carboxyl-terminal propeptide is necessary for proper sorting of barley lectin to vacuoles of tobacco. Plant Cell 1990 2:1145-1155.
- 15 4) Beuning L. L., Spriggs T. W., and Christeller J. T. (1994) Evolution of the Proteinase Inhibitor I Family and Apparent Lack of Hypervariability in the Proteinase Contact Loop. J. Mol. Evol. 39 (6): 644-654.
- 20 5) Boulter, D. (1993). Review Article Number 86. Insect Pest Control by Copying Nature using Genetically Engineered Crops. Phytochemistry 34: 1453-1466.
- 6) Broodryk, S.W. 1971. Ecological investigations on the potato tuber moth, *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae). Phytophylactica 3: 73-84.
25
- 7) Chrispeels, M.J. (1991). Sorting of Proteins in the Secretory System. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42: 21-53.
- 30 8) Cleveland, T.E., Thornburg, R.W., and Ryan, C.A. (1987). Molecular Characterization of Wound-inducible Inhibitor I Gene from Potato and the Processing of its mRNA and Protein. Plant Mol. Biol. 8: 199-207.
- 35 9) Dadd, R.H. (1985). Nutrition:Organisms In: Comprehensive Insect Physiology, Biochemistry and Pharmacology (Kerkut G.A. and Gilbert, L.L.) Pergamon Press, NY Vol 4, p313-390.
- 10) Ditta G., Stanfield S., Corbin D., and Helinski D.R. (1980) Broad Host Range

DNA Cloning System for Gram-Negative Bacteria: Construction of a Gene Bank of *Rhizobium meliloti*. Proc Natl Acad Sci USA 77 (12): 7347-7351.

- 5 11) Gleave A.P. (1992) A Versatile Binary Vector System with a T-DNA Organisational Structure Conducive to Efficient Integration of Cloned DNA into the Plant Genome. Plant Mol. Biol. 20 (6): 1203-1207.
- 10 12) Gope M. L., Keinanen R. A., Kristo P.A., Conneely O. M., Beattie W. G., Zarucki-Schulz T., O'Malley B. W., and Kulomaa M. S. (1987) Molecular Cloning of the Chicken Avidin cDNA. Nucleic Acids Res. 15 (8): 3595-3606.
- 15 13) Graham, J.S., Pearce, G., Merryweather, J., Titani, K., Ericsson L.H., and Ryan, C.A. (1985). Wound-induced Proteinase Inhibitors from Tomato Leaves. J. Biol. Chem. 260: 6561-6564.
- 20 14) Holwerda BC, Padgett HS, Rogers JC (1992) Proaleurain vacuolar targeting is mediated by short contiguous peptide interactions. Plant Cell 4:307-318.
- 25 15) James, C. and A.F. Krattiger (1996). Global Review of the Field Testing and Commercialisation of Transgenic Plants, 1986 to 1995: The First Decade of Crop Biotechnology. International Service for the Acquisition of Agri-Biotech Applications (ISAAA) Briefs No. 1. 11) ISAAA: Ithaca, NY pp31.
- 30 16) Matsuoka, K., Matsumoto, S., Hattori, T., Machida, Y., and Nakamura, K. (1990) Vacuolar Targeting and Posttranslational Processing of the Precursor to the Sweet Potato Tuberous Root Storage Protein in Heterologous Plant Cells. J. Biol. Chem. 265: 19750-19757.
- 35 17) Meisner, J., K.R.S. Ascher, and D. Lowie. 1974. Phagostimulants for the larva of the potato tuber moth, *Gnorimoschema operculella* Zell. Z. Angew. Entomol. 77: 77-106.
- 18) Michelmores, R., Marsh, E., Seely, S., and Benoit, L. (1987). Transformation of lettuce (*Lactuca sativa*) mediated by *Agrobacterium tumefaciens*. Plant Cell Reports 6: 439-442.
- 19) Moore D.S., and Michael S. F. (1995) Mutagenesis of Amplified DNA Sequences

Using Ampligase Thermostable DNA Ligase. *Epicentre Forum* 2 (4): 4-5.

- 20) Morgan, T.D., B. Oppert, T.H. Czapala, and K.J. Kramer (1993). Avidin and Streptavidin as Insecticidal and Growth Inhibiting Dietary Proteins
5 *Entomol.exp.appl.* 69: 97-108.
- 21) Murray C. and Christeller J. T. (1994) Genomic Nucleotide Sequence of a Proteinase Inhibitor II Gene. *Plant Physiol.* 106: 1681.
- 10 22) Nakamura, K., and Matsuoka, K. (1993) Protein Targeting to the Vacuole in Plant Cells. *Plant Physiol.* 101: 1-5.
- 23) Neuhaus JM, Sticher L, Meins F Jr, Boller T (1991) A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. *Proc Natl Acad Sci U S A* 88:10362-10366.
15
- 24) Saalbach G, Rosso M, Schumann U (1996) The vacuolar targeting signal of the 2S albumin from Brazil nut resides at the C terminus and involves the C-terminal propeptide as an essential element. *Plant Physiol* 112:975-985.
20
- 25) Sanchez-Serrano, J., Schmidt, R., Schell. J., and Willmitzer, L. (1986). Nucleotide Sequence of Proteinase Inhibitor II Encoding cDNA of Potato (*Solanum tuberosum*) and its Mode of Expression. *Mol. Gen. Genet.* 203: 15-20.
- 25 26) Seshagiri PB, Adiga PR (1987) Isolation and characterisation of a biotin-binding protein from the pregnant-rat serum and comparison with that from the chicken egg-yolk. *Biochim Biophys Acta* 916:474-481.
- 27) Subramanian N, Adiga PR (1995) Simultaneous purification of biotin-binding proteins-I and -II from chicken egg yolk and their characterization. *Biochem J* 308:573-577.
30
- 28) Tague BW, Dickinson CD, Chrispeels MJ (1990) A short domain of the plant vacuolar protein phytohemagglutinin targets invertase to the yeast vacuole. *Plant Cell* 2:533-546.
35
- 29) Thompson L. D. and Weber P. C. (1993) Construction and Expression of a

- Synthetic Streptavidin-Encoding Gene in *Escherichia coli*. Gene 136: 243-246
- 5 30) Torres, C., Cantliffe, D.J., Laughner, B., Bieniek, M., Nagata, R., Ashraf, M. and R.J. Ferl (1993). Stable transformation of lettuce cultivar South Bay from cotyledon explants. Plant Cell, Tissue and Organ Culture 34: 279-285.
- 31) Vitale A, Chrispeels MJ (1992) Sorting of proteins to the vacuoles of plant cell Bioassays 14:151-160.
- 10 32) Von Heijne, G. (1983). Patterns of Amino Acids Near Signal-Sequence Cleavage Sites. Eur. J. Biochem. 133: 17-21.
- 15 33) Walker-Simmons, M., and Ryan, C.A. (1977). Immunological Identification of Proteinase Inhibitors I and II in Isolated Tomato Leaf Vacuoles. Plant Physiol. 60 61-63.
- 34) Zhang, X., and Conner, A.J. (1992). Genotypic effects on tissue culture response of lettuce cotyledons. J. Genet. and Breed. 46: 287-290.
- 20 35) Synthesis and structure determination of NMR of a putative vacuolar targeting peptide and model of proteinase inhibitor from *Nicotiana glauca*. Biochemistry. 1996 Jan 16; 35(2): 369-378.
- 25 36) Molecular characterization of a novel tobacco pathogenesis-related (PR) protein: a new plant chitinase/lysozyme. Mol. Gen. Genet. 1994 Oct 28; 245(2): 246-254.
- 37) Proaleurain vacuolar targeting is mediated by short contiguous peptide interactions. Plant Cell. 1992 Mar; 4(3): 307-318.
- 30 38) A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. Proc. Natl. Acad. Sci. USA. 1991 Nov 15; 88(22): 10362-10366.
- 35 39) Conserved amino acid sequences among plant proteins sorted to protein bodies and plant vacuoles. Can they play a role in protein sorting? Eur. J. Biochem. 1991 Jul 15; 199(2): 441-450.

- 40) The vacuolar targeting signal of the 2S albumin from Brazil nut resides at the C terminus and involves the C-terminal propeptide as an essential element. Plant Physiol. 1996 Nov; 112(3): 975-985.
- 5 41) Protein targeting to the plant vacuole – a historical perspective. Braz J. Med. Biol. Res. 1996 Apr; 29(4): 413-430. Review.
- 42) Sorting of proteins to the vacuoles of plant cells. Bioessays. 1992 Mar; 14(3): 151-160. Review.
- 10 43) Short peptide domains target proteins to plant vacuoles. Cell. 1992 Feb 21; 68(4): 613-616. Review.
- 15 44) Colocalization of barley lectin and sporamin in vacuoles of transgenic tobacco plants. Plant Physiol. 1993 Feb; 101(2): 451-458.
- 45) Purification and initial characterization of a potential plant vacuolar targeting receptor. Proc. Natl. Acad. Sci. USA. 1994 Apr 12; 91(8): 3403-3407.
- 20 46) Protein transport via amino-terminal targeting sequences: common themes in diverse systems. Mol. Membr. Biol. 1995 Oct; 12(4): 295-307. Review.

All references are incorporated herein by reference.

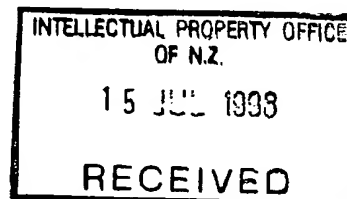
JOHN CHRISTELLER, PAUL SUTHERLAND,
COLLEEN MURRAY, NGAIRE MARKWICK
and MARGARET PHUNG

By the authorised agents

A. J. Park & Son

Per

A. J. Park



Sal I Bam H I
 | |
 ----- pUC 19
 |
 Xba I

```

1  ATGGAGTCAA AGTTTGCTCA CATCATTGTT TTCTTTCTTC TTGCAACTTC
    original sequence - ag a
51  CTTTGAAACT CTCTTGGCAC GAAAAGAAAG Tcatggacca cagatcttag
    mutagenic primer
101 aacTTCAAAA GGAATTTGAA TGCAATGGAA AACAAAGGTG GCCAGAACTT
151 ATTGGTGTAC CAACAAAGCT TGCTAAGGGG ATAATTGAGA AGGAAAATTC
201 ACTCATAACT AATGTTTCTG TACTACTGAA TGGTTCTCCA GTCACAATGG
251 ATTATCGTTG TAATCGAGTT CGTCTTTTGG ATAACATTTT GGGTGATGTT
301 GTACAAATTC CTAGGGTGGC TTA
  
```

Figure 1

```

1  GAATTCGGCA AGGaccacac gggctctctc acggcCTGCA GAGATGGTGC
    upstream primer
51  ACGCAACCTC CCGGCTGCTG CTGCTGCTGC TGCTCAGCCT GGCTCTGGTg
    cc - original sequence
101 ggggggggga tgcttccac AAAGTGCTCG CTGACTGGGA AATGGACCAA
    mutagenic primer
151 CGATCTGGGC TCCAACATGA CCATCGGGGC TGTGAACAGC AGAGGTGAAT
201 TCACAGGCAC CTACATCACA GCGTAACAG CCACATCAAA TGAGATCAAA
251 GAGTCACCAC TGCATGGGAC ACAAACACC ATCAACAAGA GGACCCAGCC
301 CACCTTTGGC TTCACCGTCA ATTGGAAGTT TTCAGAGTCC ACCACTGTCT
351 TCACGGGGCA GTGCTTCATA GACAGGAATG GGAAGGAGGT CCTGAAGACC
401 ATGTGGCTGC TGCGGTCAAG TGTTAATGAC ATTGGTGATG ACTGGAAAGC
451 TACCAGGGTC GGCATCAACA TCTTCACTCG CCTGCCACAC CAGAAGGAGT
501 GAGGATGGCC CCGCAAAGCC AGCAACAATG CCGGAGTGCT GACACTGCTT
    Hind III
531 GTGATATTCC TCCCAATTA AGCTTG
  
```

Figure 2

EcoR I
 1 GAATTCGCAT ATGGCTGAAG CTGGTATCAC CGGTACTTGG TACAACCAGC
 51 TGGGGTCTAC CTTTCATCGTT ACCGCTGGTG CTGACGGTGC ACTGACCGGT
 101 ACTTACGAAA GCGCTGTTGG TAACGCTGAA AGCCGTTATG TTCTGACCGG
 151 TCGTTACGAC TCTGCTCCGG CTACCGACGG TTCTGGTACT GCTCTGGGTT
 201 GGACCGTTGC TTGGA AAAAC AACTACCGTA ACGCTCACTC TGCTACCACC
 251 TGGTCTGGCC AGTACGTTGG TGGTGCTGAA GCTCGTATCA ACACCCAGTG
 301 GCTGCTGACC TCTGGTACCA CCGAAGCTAA CGCTTGAAA TCTACCCTGG
 351 TTGGTCACGA CACGTTCCACC AAAGTTAAAC CGTCTGCTGC TTCTATCTAGA
 Xba I

Figure 3

Sal I altered Bam H I*
 ----- - pUC 19
 Xba I

1 ATGGATGTTT ACAAGGAAGT TAATTTTCGTT GCTTACCTAC TAATTGTTCT
 51 TGGTAAGATT TTCCTTTACT CCTTTGTTTT AAAAAATAAA AAAACAAAAA
 101 AAATCTTGGT TTATACATAT ATATACACAC AAGTAGTTTT ATTTTTTTTCC
 151 TTTATATTAT ATTTGTTGTA GGAATATTTT TACTTGTTAG CGTGGTGGA
 201 CATGTTGATG CGAAGATCTG TACTAAAGAA TGTGGTAATC TTGGGTTTGG
 251 GATATGCCCC CGTTCAGAAG GAAGTCCGAA AAATCCCATATA TGCATCAATT
 301 GTTGCTCAGG CTATAAGGGT TGTAATTATT ATAGTGTTTT CGGGAGATTT
 351 ATTTGCGAAG GAGAATCTGA CCTAAAAAAC CAAAAGCTT GCCCCCTAAA
 401 TTGTGATACA AATATTGCCT ATTCAAGATG CCCCCATTCA GAAGGAAAAT
 451 CGCTAATTTA TCCCACCGGA TGTACCACAT GTTGACACAGG GTACAAGGGT
 501 TGCTACTATT TCGGTAAAAA TGGCAAGTTT GTATGCGAAG GAGAGAGTGA
 551 TGAACCCAAG GCAAATATGT ACCCTGCAAT GTGA

Figure 4

Sal I* Bgl II**

ATG . . .

↑

PPI-I signal sequence

BamH I**

Hind III

TGA

↑
Avidin mature protein cDNA

Xho I*

HindIII

NotI

CamV 35S

pART7 vector body

NotI

ocs 3'

```
graph LR
    subgraph Vector_Map [pART7 vector body map]
        direction LR
        XhoI["Xho I*"]
        CamV35S["CamV 35S"]
        pART7Body["pART7 vector body"]
        ocs3p["ocs 3'"]
        HindIII["HindIII"]
        NotI1["NotI"]
        NotI2["NotI"]
        
        XhoI --- CamV35S
        CamV35S --- NotI1
        NotI1 --- pART7Body
        pART7Body --- NotI2
        NotI2 --- ocs3p
        ocs3p --- HindIII
    end
```

Figure 5

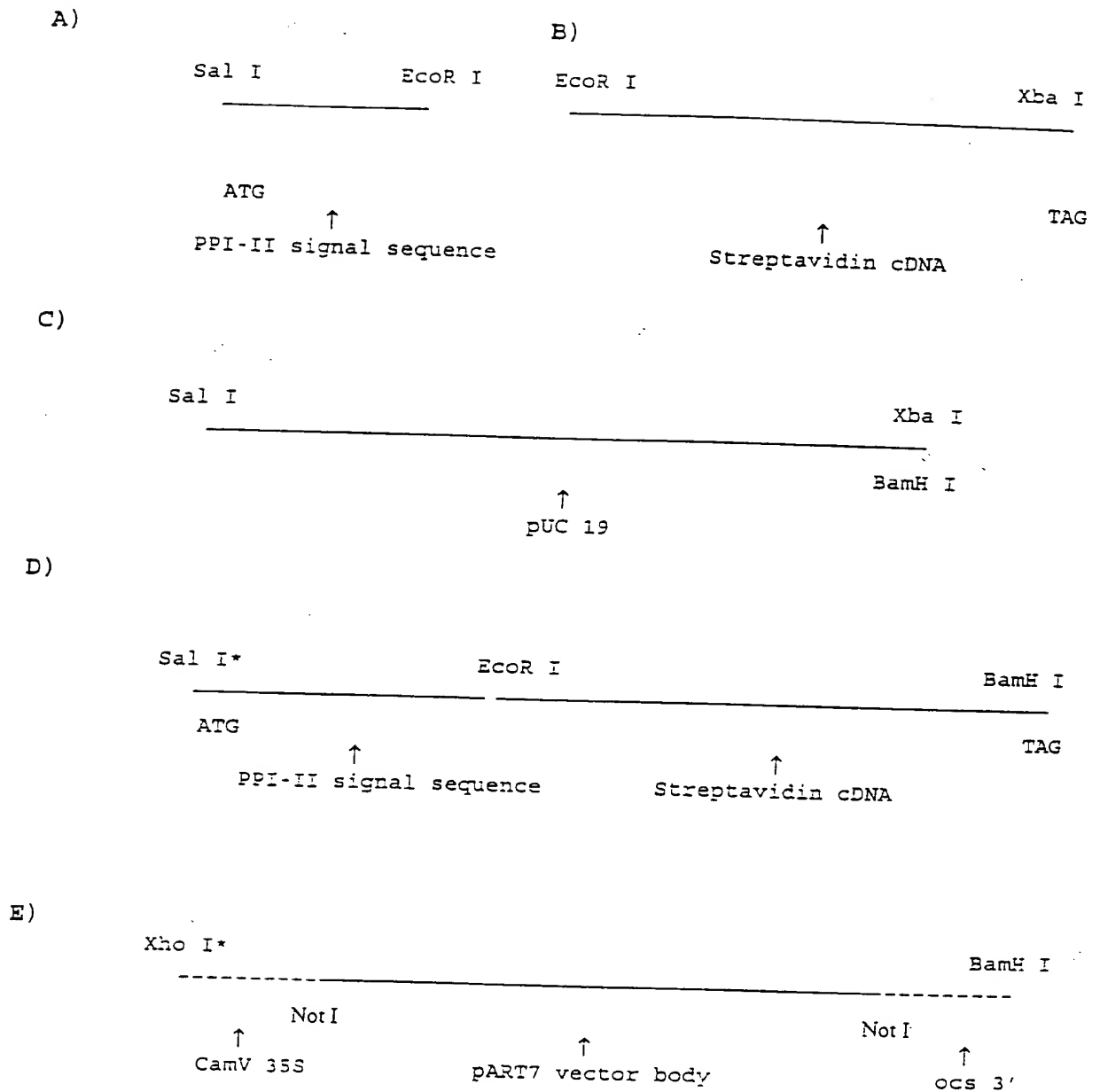
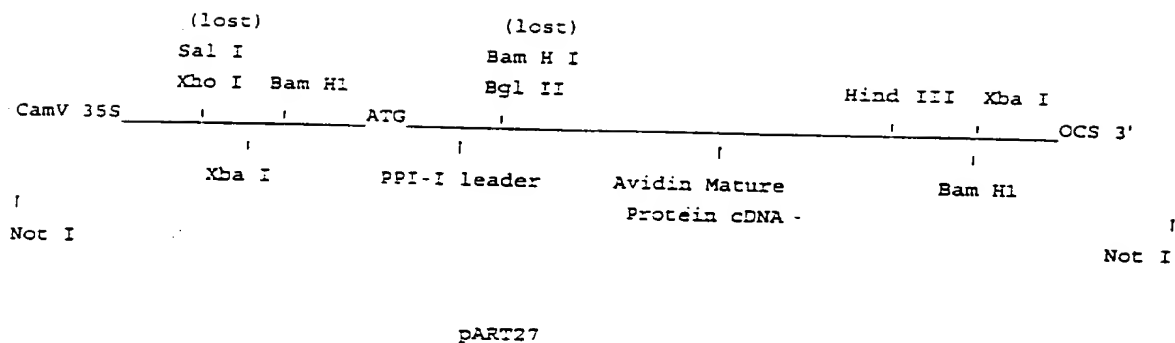


Figure 6

A)



B)

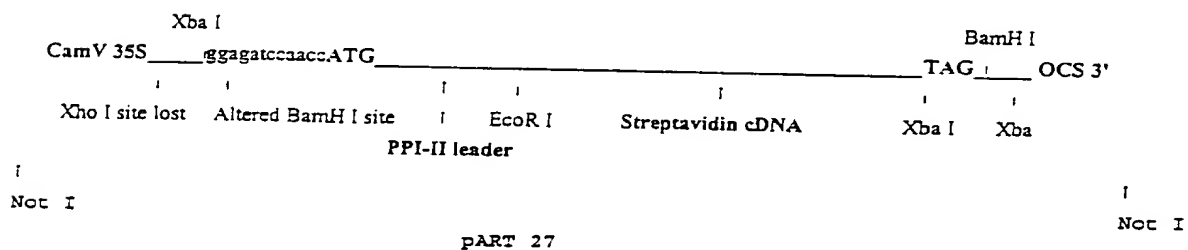


Figure 7

A)

```
1  ATGGAGTCAA AGTTTGCTCA CATCATTGTT TTCTTTCTTC TTGCAACTCC
51  CTTTGAAACT CTCTTGGCAC GAAAAGAAAG TGATGGACCA GAGATCCCTG
101 CCAGAAAGTG CTCGCTGACT GGGAAATGGA CCAACGATCT GGGCTCCAAC
151 ATGACCATCG GGGCTGTGAA CAGCAGAGGT GAATTCACAG GCACCTACAT
201 CACAGCCGTA ACAGCCACAT CAAATGAGAT CAAAGAGTCA CCATTGCATG
251 GGACACAAAA CACCATCAAC AAGAGGACCC AGCCACCTT TGGCTTCACC
301 GTCAATTGGA AGTTTTCAGA GTCCACCACT GTCTTCACGG GCCAGTGCTT
351 CATAGACAGG AATGGGAAGG AGGTCCTGAA GACCATGTGG CTGCTGCGGT
401 CAAGTGTTAA TGACATTGGT GATGACTGGA AAGCTACCAG GGTGCGCATC
451 AACATCTTCA CTCGCCTGCG CACACAGAAG GAGTGA
```

B)

```
                                cleavage site
                                ↓
1  MBSKFAHIV FFLATPPET LLARKESDGP EipARKCSLT GKWTNDLGSN
51  MTIGAVNSRG EFTGTVITAV TATSNEIKES PLHGTQNTIN KRTQPTFGFT
101 VNWKFSSESTT VFTGQCFIDR NGKEVLKTMW LLRSSVNDIG DDWKATRVGI
151 NIFTRLRTQK E*
```

Figure 8

A)

```
1  ATGGATGTTT ACAAGGAAGT TAATTTGTTT GCTTACCTAC TAATTGTTCT
51  TGGTAAGATT TTCCTTTACT CCTTTGTTTT AAAAAATAAA AAAACAAAAA
101 AAATCTTGGT TTATACATAT ATATACACAC AAGTAGTTTT ATTTTTTTCC
151 TTTATATTAT ATTTGTTGTA GGAATATTTT TACTTGTTAG CGTGGTGGAA
201 CATGTTGATG CGAAGATCTG TACTAAGAAT TCGCATATGG CTGAAGCTGG
251 TATCACCGGT ACTTGGTACA ACCAGCTGGG GTCTACCTTC ATCGTTACCG
301 CTGGTGCTGA CGGTGCACTG ACCGGTACTT ACGAAAGCGC TGTTGGTAAC
351 GCTGAAAGCC GTTATGTTCT GACCGGTCGT TACGACTCTG CTCCGGCTAC
401 CGACGGTTCT GGTACTGCTC TGGGTTGGAC CGTTGCTTGG AAAAACAAC
451 ACCGTAACGC TCACTCTGCT ACCACCTGGT CTGGCCAGTA CGTTGGTGGT
501 GCTGAAGCTC GTATCAACAC CCAGTGGCTG CTGACCTCTG GTACCACCGA
551 AGCTAACGCT TGGAAATCTA CCCTGGTTGG TCACNACACG TTCACCAAAG
601 TTAAACCGTC TGCTGCTTCT ATCTAG
```

B)

```
                                cleavage site
                                !
1  MDVHKEVNFV AYLLIVLGIF LLVSVVEHVD AKICTKishM AEAGITGTWY
51  NQLGSTFIIV AGADGALTGT YESAVGNAES RYVLTGRYDS APATDGSSTA
101 LGWTVAWKNN YRNAHSATTW SGQYVGGAEA RINTQWLLTS GTTEANAWKS
151 TLVGHDTFTK VKPSAASI*
```

Figure 9

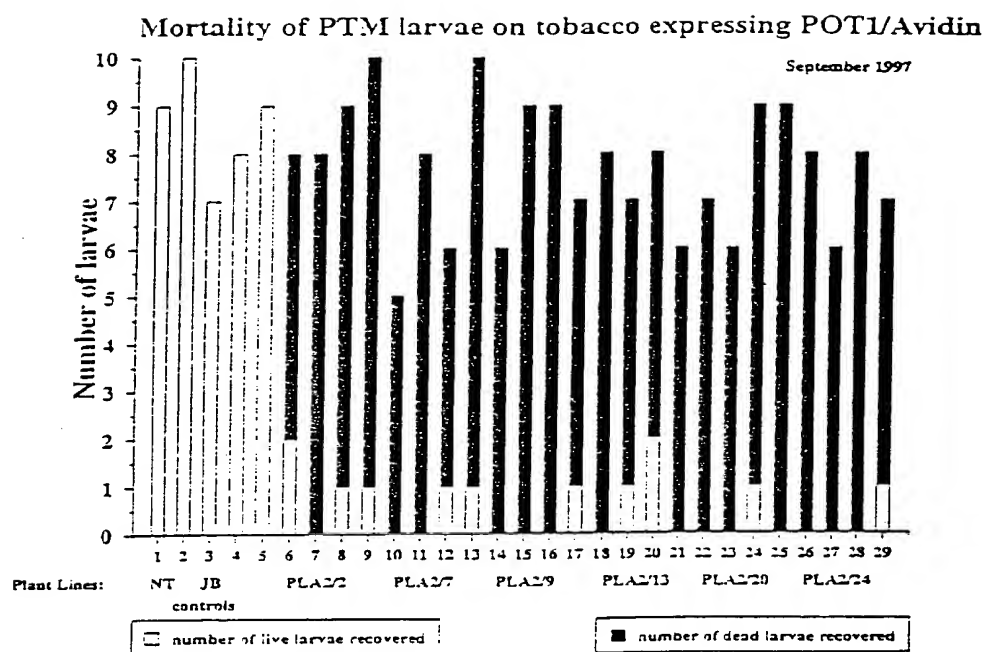


Figure 10

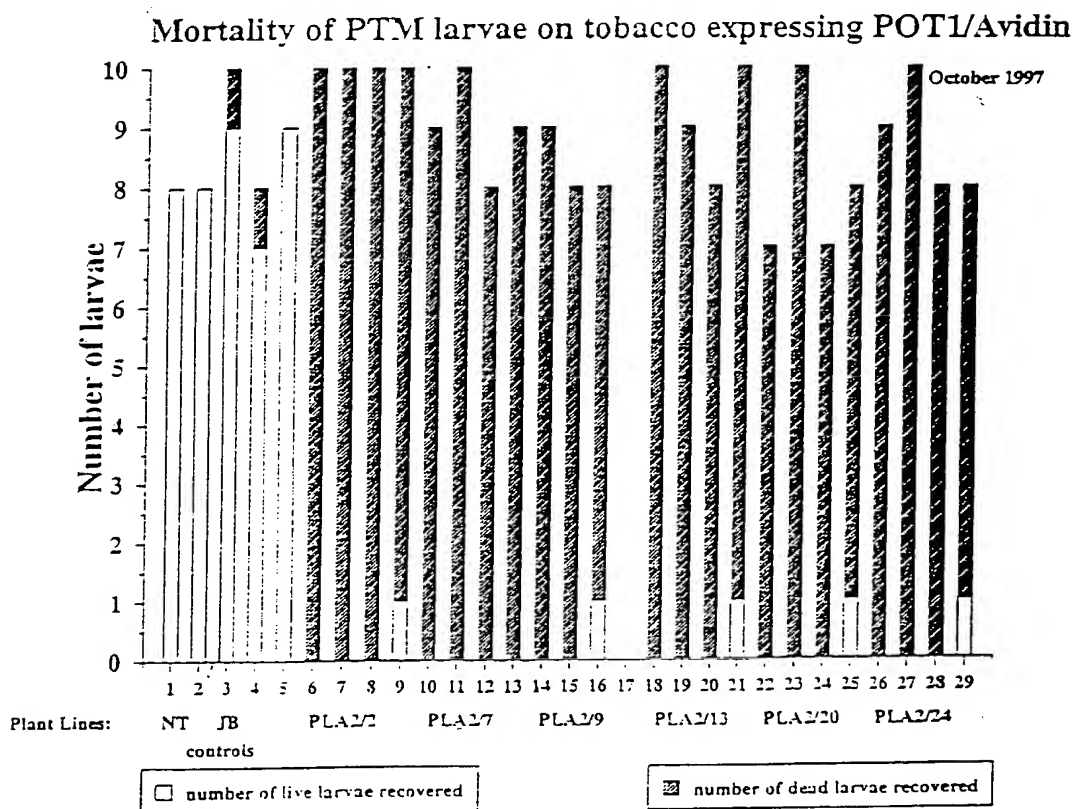


Figure 11

A)

1 CCCTCCGTCC CCGCCGGGCA ACAACTAGGG AGTATTTTTC GTGTCTCACA
51 TCGCAAGAT CGTCGTTGCA GCCATCGCCG TTTCCCTGAC CACGGTCTCG
101 ATTACGGCCA GCGCTTCGGC AGACCCCTCC AAGGACTCGA AGGCCCAGGT
151 CTCGGCCGCC GAGGCCGGCA TCACCGGCAC CTGGTACAAC CAGCTCGGCT
201 CGACCTTCAT CGTGACCGCG GGCGCCGACG GCGCCCTGAC CGGAACCTAC
251 GAGTCGGCCG TCGGCAACGC CGAGAGCCGC TACGTCCTGA CCGGTCGTTA
301 CGACAGCGCC CCGGCACCG ACGGCAGCGG CACCGCCCTC GGTGGACGG
351 TGGCCTGGAA GAATAACTAC CGCAACGCCC ACTCCGCGAC CACGTGGAGC
401 GGCCAGTAGC TCGGCGGGCG CGAGGCGAGG ATCAACACCC AGTGGCTGCT
451 GACCTCCGGC ACCACCGAGG CCAACGCCTG GAAGTCCACG CTGGTCGGCC
501 ACGACACCTT CACCAAGGTG AAGCCGTCCG CCGCCTCCAT CGACGCGGCG
551 AAGAAGGCCG GCGTCAACAA CGGCAACCCG CTCGACGCCG TTCAGCAGTA
601 GTCGCGTCCC GGCACCGGCG GGTGCCGGGA CCTCGGCC

B)

1 MRKIVVAAIA VSLTTVSITA SASADPSKDS KQVSAAEAG ITGTWYNQLG
51 STFIVTAGAD GALTGTYESA VGNAESRYVL TGRYDSAPAT DSGGTALGWT
101 VAWKNNYRNA HSATTWSGQY VGGAEARINT QWLLTSGTTE ANAWKSTLVG
151 HDTFTKVKPS AASIDAAKKA GVNNGNPLDA VQQ

Figure 12

Folder

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